SHOOT-TIP CRYOPRESERVATION MANUAL

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CHAPTER 1

INTRODUCTION: LONG-TERM STORAGE CONSIDERATIONS

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Cryopreservation is considered the best option for the long-term storage of clonal germplasm (6). Cryopreservation techniques developed over the last 25 years are now advanced to the stage where they can be implemented for useful storage of germplasm. Techniques of controlled freezing, vitrification, encapsulation-dehydration, dormant bud preservation, and combinations of these are now available for use with plant genotypes in hundreds of species. However, few labs are actually putting the techniques to large-scale use. Initial implementation of procedures can be daunting for facilities that are already low on financial and human resources. The steps of experimental protocols tested on one or a few genotypes in a genus may not work for the range of germplasm available in national or even breeder collections. Initial steps must be taken to adapt these protocols and set up procedures for testing, screening, and ultimately storing the range of genotypes in each collection (26). Although testing to date has shown no variation due to cryopreservation protocols, additional testing over time is needed to determine the long-term stability of the system.

Present day technology and the large base of research into cryopreservation methods over the last 25 years have produced several protocols for the preservation of apical shoot tips (6).

1. **Controlled Freezing** (slow cooling, two-step freezing). Shoot tips are pretreated in cryoprotectants and frozen at < 1°C/ min to about -40° C, then plunged into liquid nitrogen (18, 24, 31).
2. **Vitrification** (rapid cooling). Several protocols and cryoprotectants are available. After a relatively brief cryoprotectant pretreatment the vials are directly plunged into liquid nitrogen (29, 37).
3. **Dehydration-Encapsulation.** Apices or stem tips are encapsulated in alginate and dehydrated osmotically and with airflow before direct immersion in liquid nitrogen (5).
4. **Combinations** of the above techniques are also available (30).
5. **Dormant bud preservation.** Dormant wood collected from trees during the coldest winter months are given additional chilling, dehydrated gradually by slow cooling, and held in LN vapor. Thawed buds are grafted or budded onto rootstocks for recovery (7).

Issues Involved in Implementing LN Storage

Initial decisions

Now that many successful techniques are available and already tested on numerous genotypes, the time has come to start storing germplasm. Implementing storage requires many considerations. The present selection of cryopreserved plants vary from randomly chosen selections to carefully selected clones that represent morphological variation and unique geographic criteria sometimes referred to as "core" collections (9, 16, 26, 28). The curatorial decision regarding what to store generally starts with at-risk plants i.e. those that are likely to be lost to disease, insect pests, or environmental conditions at the field site. These decisions will vary with crop type, location of the field genebank, and economic importance of each crop. The form of plant to store is also an issue, and pollen, shoot apices, dormant buds, excised embryonic axes, or somatic embryos may be the form of choice depending on the species involved (1). The next decisions involve logistics of storage. How many samples of each accession should be stored? The number of propagules will vary with the plant type and the recovery potential. Ideally enough propagules should be stored in each container to produce several living plants and enough containers to allow for several recoveries over time. If periodic testing is anticipated, then additional vials should be included for that purpose so that the vials in the base collection remain untouched (see long-term monitoring below).

Developing a testing and storage protocol

Once the initial decisions are made on accessions to be stored and how many of each, the testing protocol must be established. The amount of initial testing varies greatly from facility to facility. If methods development involved several species and cultivars, then less testing may be needed at the storage phase. There are two schools of thought on testing. The first system uses several storage trials and their controls as the testing phase. In this scenario a lab might store five vials of 10 propagules and use one as the control. If the control recovers with a high percentage, then four vials remain in storage. If recovery of the control is low, then another group is stored to increase the number of viable propagules in storage (32). The second method stores a small number of propagules in two vials and thaws both after a short time. If the regrowth percentage is >40%, then another group is processed and stored. If the percentage is lower, improvements are made to the culture or cryopreservation protocols to improve performance before storage (9, 26).

Both internal and external controls are needed for any storage protocol. Internal controls include regrowth of plants at critical stages of the protocol. For meristem cryopreservation this might occur after dissection from the mother plant, after cryoprotection, and after LN exposure. If cryopreserved vials are transferred from one storage container to another or from one location to another, a control vial should be regrown to monitor the effect of those transfers. Proper techniques for inserting and

removing vials from storage containers should be developed before storage begins. Improper handling at the storage or removal point can easily kill propagules.

Storage location

For long-term storage the physical location of the storage dewar is very important. As a base collection of important germplasm, the dewar should be located at a site remote from the field genebank if at all possible. The storage site needs to be secure and under the control of dependable management to insure that LN is added to the dewar as needed. Alarms should be installed to monitor the LN level in the dewars and ensure constant temperature in the storage container. The use of a remote storage location is especially important when field collections are at risk due to environmental or political problems (earthquake, hurricane, flood, volcano, or civil unrest).

Long-term monitoring

Monitoring of viability of cryopreserved collections is problematic. Ideally storage would be in a dewar that is not disturbed, filled regularly, and accessed only rarely. In this ideal case viability should remain similar to control percentages and little testing is required. If storage is in a general purpose dewar that is often used, regular monitoring may be required. Since little data is available on the effect of dewar use on the life of stored propagules, it is difficult to propose an adequate testing scheme. The best advice might be to designate a dewar for storage and ensure that it is not used for other purposes. Dewars with long holding times (one to two years) are now available and would be ideal for base collections.

Cryopreservation and storage records:

Germplasm systems require information on the origin of each plant (passport information), and cryopreserved propagules must be linked to that original plant accession. Important cryogenic information must be linked as well since the propagules are to be retrieved 50, 100, or 500 years in the future. Each accession must have information on preparation, pretreatment, cryopreservation method, thawing method, and the recovery medium. Especially critical to recovering the germplasm are thawing methods and recovery media. These two items should be readily accessible in the accession database for easy access by future curators wishing to recover plants. Complete protocol information could be in a secondary database as it is not critical to recovering plants, but may be of scientific interest.

Long-Term Storage Stability

Plant tissues were first cryostored in the 1970's and initial tests with short-term storage of pea (2 yr), strawberry (10 yr) and potato (1 yr) shoot tips indicate no loss of viability (14, 19, 34). Kartha et al. noted fluctuations in strawberry meristem viability after various LN storage periods, but were attributed to differences in the physiological status of the shoot tips used in different experiments, not the result of LN storage. Three generations of field grown strawberries produced runners, exhibited vigorous growth, and produced normal fruit (17). Towill (34) recovered normal potato plants from cryopreserved shoot tips (34) and Reed et al. (25, 27) observed that strawberry plants of 10 cultivars stored in LN and evaluated in the field all produced normal leaves, flowers, and fruit.

Phenotypic changes arising in plants grown in tissue culture are termed somaclonal variants (21). These variations are most common in plants regenerated from single cells or callus. Variation occurs, but is not common, in micropropagated or meristem propagated plants derived from existing shoot tips. If care is taken in the clonal multiplication of plant materials, the amount of variation in propagules should be no different from field plants. Genotypes that often produce mutations/sports will also produce them in vitro (banana is a good example of a variable genus). Germplasm scientists who are familiar with the plants to be cryopreserved are able to recognize genetic variability before or after the cryopreservation process. The establishment of descriptor characteristics for each genotype allows for rouging of off types upon regrowth. Any genotype with a known tendency to produce sports/mutations should be flagged in the database and propagules carefully selected for storage. Field and genetic analyses are needed to determine whether instability is a problem, however several studies have shown little need for concern (10-13, 20, 35).

Physical and Biochemical Stability

Another factor relating to viability of propagules stored for long periods involves the physical and biochemical stability of the system. Vitrified solutions are known to crack from physical shocks or in response to certain warming procedures. Thermal-stress induced fractures of biological materials may cause serious damage to stored samples. Slow cooling rates minimize thermal stress from non-uniform temperature distribution, and cryoprotectants reduce stress by changing the microstructure of the ice formed (8). Fractures can also occur as random events during cryopreservation (22). Fractures typically occur in large organs such as whole seeds and are less common in cell suspensions and shoot tips. Most reports of physical cracking are with animal organs (liver slices, veins, and arteries) rather than plant specimens.

The kinetic stability of red blood cells and liposomes was found to decrease at a predictable rate if stored at temperatures above the glass transition (33). The stability of vitrified biological materials held at temperatures above the glass transition was predicted to decrease by a factor of 10 for every 15° C. As a glassy (vitrified) system cools toward its glass transition temperature, cellular processes become increasingly slow or nearly

arrested because of high viscosity. Molecular movement is also slowed. Additional studies of the relationship between the glass transition temperature and the stability of cryopreserved organisms are needed to adequately predict storage life of biological collections (33).

Cryopreserved samples held in dewars are exposed to warming and cooling cycles as other samples are added or removed. There are no studies that quantify the effects of these temperature variations on storage life. Possibly we could apply the stability principles discussed above to this phenomenon and hypothesize that these fluctuations in temperature would impact the storage life of a cryopreserved sample. It is also not known if stability varies among the different cryopreservation procedures.

Biochemical stability of cryopreserved plant cells is confirmed by the analysis of many cell cultures producing secondary products (2, 3, 38). Studies of the genetic stability of cell and meristem cultures also validate the stability of those systems (10-13).

Table 1. Advantages and disadvantages of some commonly used cryopreservation techniques.

Technique	Advantages	Disadvantages
Controlled cooling	Stability from cracking relatively nontoxic cryoprotectants takes little technician time	Requires equipment, slow recovery, low applicability to tropical species.
Vitrification	No special equipment needed, fast procedure, fast recovery	Vitrification solutions are toxic to many plants, cracking is possible, requires careful timing of solution changes.
Encapsulation-dehydration	No special equipment needed, nontoxic cryoprotectants, simple thawing procedures	Requires handling each bead several times, some plants do not tolerate the high sucrose concentrations.
Dormant bud preservation	Easy, useful for many temperate tree species	Requires freezing equipment, larger storage space, recovery requires grafting or budding, works best in cold temperate regions.

Is One Protocol Better Than The Others?

Some of the relative advantages and disadvantages of cryopreservation protocols for long-term storage are evident (Table 1), however many may not be evident for many years. At present the choice of techniques is related to the needs of the facility involved. Personnel, equipment, expertise, plant type, and available facilities may influence which technique is most appropriate.

Germplasm storage

Curators will need to determine the amounts and types of germplasm to be stored based on plant characteristics. A rough estimate of the number of propagules needed should be based on the percent recovery following cryopreservation, the ease of regrowth of the plant, and the number of times samples will be removed from storage. For most clonally propagated crops recovery of five or more plants from a vial would provide adequate material for micropropagation. If the cryopreserved control recovery were 50% or more, then 10 shoot tips per vial would be adequate for long-term storage. If more individuals are desired or the recovery percentage is lower, 25 shoot tips per vial might be warranted. Most accessions could be stored as four or five vials, thus allowing four or five uses over 100 years. Any accessions used for viability testing over time should include 10 or more vials.

Cryopreserved storage of important crop plants is being initiated in many countries throughout the world. The storage form varies with the crop type and the facilities available (Table 2). The numbers of accessions are low in most cases due to lack of funding for personnel to perform germplasm preservation at most sites.

Dormant branches of temperate trees are readily available from orchards and field collections. Storage of dormant wood requires more space than some other forms, however the ease of storage may make up for the space requirement. Grafting and budding skills are required for recovery of these materials. An 8-yr study of mulberry dormant buds found no change in the ability to form shoots with increased length of storage at -135°C (23). Nearly 74% of 376 mulberry cultivars preserved for 5 yr had 50% or more shoot regrowth, and only 6% had regrowth of less than 30% of buds.

In vitro shoot tips are widely tested in cryopreservation protocols because they are easy to multiply, available any time of year, easy to manipulate physically and physiologically, and can be recovered in culture. While in vitro systems require some additional input before storage, the ease of recovering and propagating the shoot tips is an advantage, and many accessions can be stored in a small dewar.

Embryonic axes are ideal storage forms for some species with recalcitrant seed. Removal and drying of the axes is time consuming, but not usually difficult, and the resulting propagules can be stored in a small space and recovered in vitro.

Somatic embryos are also a good form for cryopreserved storage. Many forest tree production systems depend on embryogenic callus to produce somatic embryos for testing. Over 5000 genotypes of 14 conifer species are cryostored in one facility in British Columbia, Canada alone. Storage of these embryos allows for continued use of a line after an extended period of testing in the field (4). Long-term sub culturing of embryogenic cultures can lead to somaclonal variation, so cryopreservation of important lines from freshly initiated lines is very important.

Pollen storage in liquid nitrogen is an important tool for plant breeders. Pollen storage requires drying pollen to low moisture contents. Pollen of many plants is easily stored and can be used for crosses that are not possible with fresh pollen due to differences in bloom time (15, 36).

Conclusions

Cryopreserved collections of clonally propagated germplasm are now in place for many important horticultural crops. These initial collections provide the first long-term storage for clonally propagated crops. Cryopreservation should be considered a backup to field collections to insure against loss. Long-term viability studies monitoring the recovery of designated accessions will provide additional information on the stability and viability of this storage form.

Curators planning cryostorage for their crops should first determine the most practical technique for their facility and the crops involved. Off site storage should be arranged well in advance with a reliable facility. A reciprocal exchange of LN-stored germplasm might be advantageous to two facilities needing off site locations. Records management should be of prime importance since the recovery of plants from cryostorage will depend on knowing the proper techniques for thawing and regrowth of each accession.

Curators interested in using cryopreservation for long-term storage of germplasm collections should consider several steps before initiating storage. First, determine the best storage form for the crop in question; second, prioritize accessions to be stored; third, determine the best technique to apply to these accessions; fourth, set up a database for needed information; fifth, make arrangements for offsite storage; sixth, plan long-term monitoring; and finally initiate long-term germplasm storage.

Table 2. Cryopreserved collections of clonally propagated plant germplasm stored as dormant buds, in vitro meristems, or excised embryonic axes.

Taxon	Country	Technique	Number Accessions/Replicates
Dormant Buds			
Apple	USA (NSSL)	E-D + CF	2100 accessions
Elm	France (AFOCEL)		101 accessions
Mulberry	Japan (NIAR)	CF	45 accessions
In Vitro Meristems			
Apple	China	CF/E-D	20 accessions/50 meristems
Blackberry	USA (NCGR)	CF	17 accessions/100 meristems
Cassava	Columbia (CIAT)	E-D	95 accessions/30 meristems
Grass	USA (NCGR)	CF/E-D	10 selections/100 meristems
Hops	USA (NCGR)	CF	2 accessions/100 meristems
Pear	USA (NCGR)	CF	106 accessions/100 meristems
Potato	Germany (DSM/FAL)	Droplet	219 accessions/40-350 meristems
	Peru (CIP)	Vit	197 accessions/250 meristems
Ribes	USA (NCGR)	Vit	5 accessions/100 meristems
	Scotland (UAD)	E-D	31 accessions/25-30 meristems
Embryonic Axes			
Almond	India (NBPGR)	D-FF	29 accessions/20 axes each
Citrus	India (NBPGR)	D-FF	12 acc of 6 species/50-100
Hazelnut	USA (NCGR)	D-FF.	5 Species/100-300 axes
Jackfruit	India (NBPGR)	D-FF/Vit	3 accessions/25 axes
Litchi	India (NBPGR)	D-FF	2 accessions/30 axes
Tea	India (NBPGR)	D-FF	85 accessions/25 axes
Trifoliate Orange	India (NBPGR)	D-FF	1 accession/30 axes

Techniques: E-D – Encapsulation-Dehydration, CF – Controlled Freezing, Vit – Vitrification, Droplet – Droplet Freezing, D-FF – Dehydration-Fast Freezing.
Facilities: NSSL –National Seed Storage Laboratory; AFOCEL-Association Forêt-Cellulose; NIAR –National Institute of Agrobiological Resources; NCGR – National Clonal Germplasm Repository-Corvallis; CIAT- International Center for Tropical Agriculture; DSM/FAL – Deutsche Sammulung von Mikroorganismen und Zellkulturen/Institute fur Pflanzenbau, Bundesforxhungsanstalt fur Landwirtschaft ;UAD - University of Abertay-Dundee; CIP – International Center for the Potato; NBPGR – National Bureau of Plant Genetic Resources.

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CHAPTER 2

SLOW-COOLING PROTOCOLS

The success of various cryopreservation techniques requires attention to critical points of the protocols. In the case of slow-cooling techniques, the type and length of cold acclimation (CA) and the cryoprotectant used are extremely important as well as the cooling rate. Deeper cold hardiness and increased regrowth in pear following cryopreservation are produced by alternating-temperature CA treatments that expose the plantlets to freezing temperatures (-1 °C) and shorter warm periods (22 °C) (2). Extended alternating-temperature CA periods of 2 or more weeks may also be needed for reaching optimum cold hardiness in some *Humulus* accessions. The efficacy of the cryoprotectant PGD as a cryoprotectant for use in slow-cooling protocols has been shown for cells and shoot tips of numerous genera (1,2,3,4,5,6,8,9). Storage of large germplasm collections require protocols that work well for the particular lab involved and that are successful for many types of plants. As germplasm curators prioritize the type and amount of germplasm to store, they also need to choose a technique that fits their facility. Personnel, equipment, expertise, plant type and available facilities influence which technique is most appropriate for a particular facility (7). When personnel are the rate limiting factor, and plants are tolerant to cold acclimation, slow cooling is a time, labor, and financially efficient method for cryopreserved storage of large numbers of accessions. Choosing a well-tested technique and applying it to a new genus can save development time and speed up storage of important plant collections.

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SLOW COOLING

(Reed, 1990)

In vitro plantlets are grown for 1-4 weeks under cold acclimation (CA) conditions (22 ^{o}C 8-h days and –1 ^{o}C 16-h nights) prior to cryopreservation. Pretreatment: Shoot tips (< 1 mm) taken from these plants are grown for two days on a firmer medium (1 g more agar than normal) with 5% DMSO added. See crop specific programs at the end of this chapter for additional details.

Materials to be prepared in advance:

1) Liquid MS medium without hormones in 50 to 75 ml containers (sterile)
2) Petri dishes with filter papers for draining shoot tips (sterile).
3) Small sterile containers for PGD cryoprotectant and liquid MS.
4) Frozen block of ice or artificial ice to set PGD and MS on during procedure
5) Cryotube container frozen in a block of ice.
6) Petri dishes of pretreatment medium (base medium, no hormones, with 5% DMSO and 1 g/L more agar).
7) Cell wells or small petri dishes with recovery medium.
8) Plants cold acclimated for 1 to 4 weeks in the CA incubator (-1°C 16-h dark/ 22°C 8-h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$).
9) Shoot tips (0.8 to 1.0 mm in size) grown on pretreatment medium (1 g more agar than normal with 5% DMSO added) for two days in CA conditions.

1. Just prior to freezing run, mix cryoprotectant solution: 50 ml total

WEAR GLOVES FOR THIS PROCEDURE AND WASH THEM BEFORE TOUCHING OTHER MATERIAL OR TELEPHONE ETC. DMSO is a solvent that can easily move other chemicals through the skin and into the blood stream.

a) in a 50 ml graduated cylinder add 30 ml of liquid MS medium (no hormones) and small stir bar.
b) add 4.9 ml DMSO (reagent grade, do not use DMSO older than 1 year).
c) slowly add 5 g glucose (dextrose) while stirring.
d) very slowly add 5 g polyethylene glycol MW10,000 (PGD) while stirring and let stir until dissolved.
e) bring to volume (50 ml) with liquid MS (NOT WATER), cover with parafilm to mix.

2. Filter-sterilize cryoprotectant through a filter flask and place the flask in the FREEZER until ready for use (at least 30 minutes but not much longer or it will freeze). Wash out the cylinder and stir bar immediately yourself, do not leave it for others. WEAR GLOVES. While the cryoprotectant is cooling you have 30 min to do steps 3 and 4.

3. Label cryovials and place in FROZEN tube container in hood. Unscrew lids but leave

them on top of vials. Add 2 drops of liquid medium using a sterile pipette. To keep the pipette sterile, place sterile pipette tip first in a sterile Petri dish with the lid covering much of the pipette. Be sure to have a control vial of PGD without shoot tips for the sample probe. It needs to be the same temperature as the vials with shoot tips.

4. Add pretreated shoot tips to the vials. Check to be sure they are in the proper vials. Over a 30 min period add the chilled cryoprotectant gradually to the shoot tips. Start with two drops every 2 minutes for 6 minutes then add 4 drops every 2 minutes. The vial should be filled to the very top when the 30 minutes are up. WEAR GLOVES

5. Place the tray with the shoot tips in the REFRIGERATOR or FREEZER for 30 minutes then remove excess PGD down to the 1 ml mark. Close lids firmly but gently, do not over-tighten or you will have a bad seal and liquid nitrogen will enter the vials.

Freezer Program:

Pyrus / Ribes/ Humulus 0.1 °C/min to –40 °C
Rubus 0.5 °C/ min to –35 °C

Set Up Program
1. 50°C/min to 0°C Cool Plus Chamber (this is to cool the chamber before you put in your samples)
2. Hold at 0°C for 2 minutes. Use this time to add your samples. Add them quickly and insert the sample probe into a vial with PGD only.
3. Speed/Temp (i.e. 0.1/-9°C) Cool Plus Sample (to cool the samples to the cryoprotectant freezing point)
4. 99.9°C/min to –50 °C Cool Plus Chamber (to cause the exotherm)
5. 20°C/min to –15 Warm Chamber (so the sample temperatures don't drop too fast)
6. Speed/Temp (i.e. 0.1/-40°C) Cool Plus Sample (to finish the cooling at the right rate)
7. End

To start the freeze.

1) Set up pen for chart paper and turn on power.
2) Start the freezer program to cool the chamber to 0°C. When the chamber reaches 0°C quickly place vials to be frozen and the vial for the sample probe (BUT NOT THE CONTROLS) in the freezing unit. When you close the door the cooling will resume

3) Return vial tray to the REFRIGERATOR for the controls to stay cold. Rinse controls and place them on cell wells <u>after the exotherm</u> but before the freezing run ends.

4) When the freezer reaches –9 ^{o}C (or the freezing point of the cryoprotectant you are using) it will seed the samples by dropping the chamber temperature to –50 ^{o}C. At this point there should be an exotherm (the sample temperature line should go up by

5 to 10 ^{o} C). If this does not occur, open the door and shake the sample tray, close the door and see if an exotherm occurs, if not repeat until it does. The sample temperature should not go below -15 °C at the lowest. The vials should be opaque as ice will have formed.

5) Don't forget to rinse and plate the controls. Follow directions 6, 7, 8 and 9 below at the time of the exotherm.

At the end of the programmed freeze:

1. Quickly transfer the vials to a dewar of liquid nitrogen or put on a cane and plunge into storage container. Cap but do not screw on lid. Let set for 10 min to 1 hr or until ready to thaw. The minimum is about 10 min, the maximum is indefinite as long nitrogen remains in the dewar.

2. To thaw, fill a 1 liter plastic beaker with hot water (45 ^{o}C) and one with cold tap water (22 °C). With long forceps quickly remove 5 vials from the nitrogen and add them to the hot water. Stir with the thermometer for exactly 1 minute then quickly scoop them up with your hand and put into the cold water. Stir.

3. Repeat until all vials are thawed. Vials can be removed from the cold water when they have thawed completely. Place vials in a tray after shaking off excess water or dry with a tissue.

4. Place vials in the hood, loosen caps. WEAR GLOVES FOR THE REST OF THIS PROCEDURE. Drain off cryoprotectant down to 0.25 ml (where the tube tapers) and fill with liquid medium (room temperature).

5. Let vials set for 5 minutes then drain off medium with a pipette, pick up shoot tips with pipette, and place on a small filter paper filled Petri dishes. Pick up shoot tips with a metal pick (be careful not to crush them) and place on growth medium in cell wells. Label cell wells and seal with Parafilm. Place on growth room shelf.

6. With gloves on, remove filter papers from Petri dishes and discard. Rinse all small glassware well and place in rack for washing. Wipe down hood.

7. Check for regrowth weekly for 6 weeks and record callus and plantlet growth

CHAPTER 3:

VITRIFICATION TECHNIQUES

Reed Vitrification Technique

(Luo and Reed, 1997, Modified from Yamada et al., 1991)

Prepare in advance:
1. Cold hardened plants for meristems. (1-4 wk at -1 °C for 16 hr/22 °C for 8 hr).
2. Petri dishes of pretreatment medium. (MS medium with 5% DMSO and 1% more agar)
3. Liquid MS medium with 0.8 M sucrose at pH 5.8. (see Appendix D)
4. Sterile containers for cryoprotectant and liquid media.
5. Liquid medium with 1.2 M sucrose at pH 5.8. (see Appendix D)
6. Sterile filter paper strips for draining meristems.
7. Recovery medium. (use standard growth medium without auxin)
8. Tube holder frozen in a block of ice (or something similar).

Dissect meristems and place on Pretreatment Medium: Standard growth medium with 5% DMSO and increased agar (1 g/L more) for two days in cold hardening incubator (-1 °C for 16 hr/22 °C for 8 hr).

On the day of experiment use **one** of these pretreatments prior to adding PVS2:
A) Presoak in 1% Bovine Serum albumen or 10% proline for 2 hour in O.4 M sucrose MS medium
Or
B) presoak in 2M glycerol and 0.4 M sucrose in MS for 20 min.

Make the cryoprotectant (PVS2) for 50 ml. (adjust volume as needed)

Glycerol	[(30g/100 ml /density 1.2613)/2= 23.8 ml/2]	11.9 ml
Ethylene Glycol	[(15 g/100 ml/density 1.1088)/2= 13.5/2]	6.8 ml
DMSO	[(15g/l00 ml/density 1.1)/2=13.6/2]	6.8 ml

up to 50 ml with liquid MS medium with 0.8 M sucrose (136.92 g/500ml) at pH 5.8

Mix and Filter sterilize cryoprotectant the same day as the experiment. Do not store it.

Freezing procedure:
1. Fill small dewar with liquid nitrogen.
2. Place 20 meristems in a cryotube with 1 ml PVS2 on ice. START TIMER
3. Hold at 0 °C for a total of 20, 25 or 30 minutes from initial contact with PVS2.
4. Submerge in LN2, one vial at a time using forceps. Hold under the surface for 15 seconds before releasing.

Thawing procedure:

1. Transfer 1 or 2 vials from LN2 to 45 °C water for 1 min with stirring.
2. Move to 25 °C water for 1-2 min.
3. Dry the outside of the tubes before opening.
2. Immediately add 1.2 M sucrose liquid medium to the top of the tube.
3. Immediately drain and replace with liquid medium 2 times.
4. Drain onto filter paper and transfer to recovery medium.

Matsumoto Vitrification Technique

Vitrification technique for tropical meristems (Matsumoto et al., 1998)

1. Preculture meristems on ½ MS medium with 0.3 M. sucrose for 16 hr at 20 °C.
2. Loading solution for 20 min at 25 °C in 2 M glycerol + 0.4 M sucrose.
3. PVS2 for 10 min at 25 °C.
4. LN
5. Rapid warming in 40 °C water
6. Loading solution for 20 min at 25 °C in 2 M glycerol + 0.4 M sucrose.
7. Plate on ½ MS medium with 0.1 mgL^{-1} BA. Use a filter paper between the meristem and the medium and transfer to new medium after one day by moving the filter paper.

Two and Three Step Vitrification

T. Matsumoto and A. Sakai. Cryopreservation of grape in vitro-cultured axillary shoot-tips by three-step vitrification, Euphytica, 2003, vol. 131, no. 3, pp. 299-304.

1. Preculture meristems on ½ MS medium with 0.3 M. sucrose for 3 days at 25 °C.
2. Loading solution for 20 min at 25 °C in 2 M glycerol + 0.4 M sucrose.
3. PVS2 for 80 min at 0 °C. (2 step procedure) or
4. 50% PVS2 for 30 min at 0 °C followed by full strength PVS2 for 50 min (3 step procedure)
5. LN
6. Rapid warming in 40 °C water
7. 1.2 M sucrose MS for 20 min at 25 °C.
8. Plate on ½ MS medium with 1 mgL^{-1} BA and 3% sucrose. Use a filter paper between the meristem and the medium and transfer to new medium after one day by moving the filter paper.

Alternate Method Combining Alginate and Vitrification

From Hirai et al 1998

Mint and Fragaria in alginate beads:

1. Make beads and do sucrose preculture as in encapsulation-dehydration procedure (Chapter 4)
2. Place beads in loading solution for 1 hr at 25 °C (2 M glycerol + 0.4 M sucrose).
3. Remove loading solution and add PVS2 for 2 hr at 0 °C
4. Immerse in LN
5. Thaw at room temperature
6. Rehydrate in 2 M glycerol + 0.4 M sucrose 2X, hold 10 min then plate.

Taro Vitrification

Cryopreservation of *in vitro*-grown shoot tips of taro (*Colocasia esculenta* (L.) Schott) by vitrification: 1. Investigation of basic conditions of the vitrification procedure
Takagi H; Thinh N T; Islam O M; Senboku T; Sakai A Japan Int. Res. Cent. Agric. Sci., Okinawa Subtrop. Stn., 1091-1 Kawarabaru, Maesato, Ishigaki City, Okinawa 907, Japan
Plant Cell Reports 16 (9). 1997. 594-599.

1. Excised shoot tips precultured on solidified MS supplemented with 0.3 M sucrose and maintained under a 16 h photoperiod at 25 °C for 16 h
2. Loaded with a mixture of 2 M glycerol with 0.4 M sucrose for 20 min at 25 °C.
3. The shoot tips were then sufficiently dehydrated with a highly concentrated vitrification solution (PVS2) for 20 min at 25 °C prior to immersion into liquid nitrogen.

Successfully vitrified and warmed shoot tips resumed growth within 7 days and developed shoots directly without intermediate callus formation. The average rate of shoot recovery amounted to around 80%, and the vitrification protocol appeared to be very promising for the cryo-preservation of taro germplasm.

CHAPTER 4

ALGINATE BEAD-DEHYDRATION

(Dereuddre et al., 1990)

Checklist for Alginate Bead Cryopreservation

Day 1: Items needed to dissect shoot tips and make beads

1. Tools
2. Sterile petri dishes
3. Sterile filter papers
4. Medium to hold shoot tips until beads are made
5. Alginate solution
6. Calcium chloride solution
7. Sterile 250 ml beakers for calcium chloride
8. Small sterile Petri dishes or beakers for alginate
9. Sterile strainers
10. 0.75 M sucrose MS solution in 125 ml flasks
11. Sterile pipettes for making beads
12. Cold acclimated plants for shoot tips

Day 2: Dry Beads and Cryopreserve

1. Sterile strainer
2. Sterile 250 ml beaker
3. Dry filter paper for draining beads
4. Sterile Petri dishes for drying beads (1 for each 25 beads)
5. Tools and sterile Petri dishes
6. Liquid medium for rehydrating beads
7. Cryotubes and markers
8. PI numbers and names of accessions
9. Cell wells for each accession

Prepare in advance:

1. Cold hardened plants or shoot tips precultured on solidified MS supplemented with 0.3 M sucrose and maintained under a 16 h photoperiod at 25 °C for 16 h
2. Agar plates for holding shoot tips temporarily.
3. Regular liquid MS for rehydrating the beads (10-25 ml). (see Appendix D)
4. Liquid MS with 0.75 M sucrose for pretreatment (75 ml in 125 ml flasks). (see Appendix D)
5. Liquid MS without calcium and with 3% low viscosity alginate and 0.75 M sucrose (in a flask). This is very difficult to dissolve, so heat medium and add alginate slowly, boil to

dissolve.

6. Liquid MS with 100 mM calcium chloride for forming beads (in a flask). (see Appendix D)
7. Sterile 250 ml beakers for forming beads in calcium chloride and for draining beads.
8. Sterile pipettes for forming beads.
9. Sterile Petri dishes for holding beads during dehydration.
10. Sterile sieves or tea strainers for removing beads from solutions
11. Petri dishes with sterile filter paper for draining beads.

You will need a separate flask of 0.75 M sucrose, 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile Petri dish and pipette per treatment (or genotype).

The procedure:

This is a two day procedure once the cold hardening is accomplished.

1. Dissect meristems onto regular plates until enough are collected.
2. Suspend in alginate solution in a small sterile beaker or Petri dish.
3. Mix meristems with alginate solution. Using a sterile pipette, drop meristems into a 250 ml beaker of liquid MS with 100 mM calcium chloride medium to make beads.
4. Leave the beads in the solution for at least 20 minutes to firm up.
5. Place beads in 0.75 M sucrose in 125 ml flasks on a shaker for 18-20 hours (In at 3 PM, out at 9 AM).
6. Drain beads and briefly place on sterile filter paper in Petri dish to absorb excess moisture.
7. Place beads in open sterile Petri dishes and dry in the air flow for 3 or 4 hours. They should not touch each other or they will not dry properly. There should be no extra moisture in the dish (absorb with sterile filter paper if necessary).
8. Place beads in cryovials and submerge in liquid nitrogen one at a time, hold under the surface for 30 seconds then release. Or place on cane and submerge.
9. Hold in LN for 10 min or more as schedules permit.
10. Warm at room temperature for 15 minutes, then add liquid MS medium to the tube for 5 minutes to rehydrate, place beads on medium to grow (meristem side down). Use a softer than normal recovery medium (1g/L less agar than regular growth medium)
11. If necessary, remove plantlets from beads after 2 weeks of growth. This should not be necessary if beads are rehydrated and grown on soft medium.

Dumet Alginate Bead Technique

This modification of the E-D procedure uses sucrose to substitute for cold acclimation.

Plant Materials: Use in-vitro grown shoots in growth-room conditions.

Pretreatment: Shoots (1 cm) with most leaves removed were cultured for 1 wk in Petri dishes containing MS medium with 0.75M sucrose.

Cryopreservation: Meristems (0.8 mm) are excised from the conditioned shoots, encapsulated in alginate droplets (low viscosity, 3% [w/v] Sigma Chemical), prepared with 0.75M MS medium free of calcium/cobalt salts), and allowed to polymerized for 10 min in a 100 mM $CaCl_2$ solution (Fabre and Dereuddre, 1990). Beads are transferred to 50 ml of 0.75M sucrose liquid MS medium (50 beads/ flask) and shaken for 20 to 22 h. After sucrose treatment beads were dried on sterile filter paper and dehydrated for 4 h under laminar flow at ambient temperature (20/25 ^{o}C). Final moisture content was about 20% on a fresh weight basis (FWB). Desiccated beads (10/tube) were transferred to 1.2 ml plastic cryotubes attached to canes, and plunged into liquid nitrogen (LN) in the storage tank Cryotubes were removed from the cryotank after 24-h storage in liquid nitrogen and warmed for 20 min at ambient temperature (20-25°C). Rehydrate beads in liquid MS medium for 5 min before plating on recovery medium. Use 1g/l less agar in recovery medium.

Controls:

1) **dissection control (10 for each experiment)**
2) **after osmotic desiccation (10 meristems)**
3) **after evaporative desiccation (10 meristems)**
4) **after liquid nitrogen (10 meristems)**

Moisture content determination of alginate beads

Whether beads are dried in air flow or over a drying agent, the final moisture content will impact the success of the process. Moisture content is expressed as a percentage of fresh weight. Final moisture contents in the range of 15-20% are most likely to be successful.

> Moisture content was determined as the difference between dry weight (DW) and fresh weight (FW) calculated as [FW-DW)/FW] x 100%. Dry weight was determined after heating the beads in an oven at 103°C for 16-24 h.

Ten beads for each drying time should be weighed in aluminum weigh boats, dried for the allotted time and weighed again, then dried in a 103° C oven for 16 h. Cool in a desiccator and reweigh.

Do a desiccation curve with the container at a set temperature and a standard quantity of silica gel to determine the optimum time for the required moisture content. Laminar flow drying will vary with room humidity and temperature as well as the rate of air flow. These parameters may change over the seasons. With a drying agent, dry beads over a standard amount of silica gel (i.e. 125 g or 300 g) for standard time periods (0-6 h). Some examples are: 40 g silica gel with 20 beads for 1-6 hr for *Actinidia*; 50 g silica gel for 3 hr for *Wasabi*. Determine moisture content.

Regenerate silica gel at 160° C for 6 h.

CHAPTER 5

DORMANT BUD PRESERVATION

Two-step cooling for dormant buds

L.E. Towill and P.L. Forsline. 1999. Cryopreservation of sour cherry (*Prunus cerasus* L.) using a dormant vegetative bud method. Cryo-Letters 20:215-222.

Scion wood twigs containing current season's growth are processed. Cold-chilling units have been met (0°C for at least 72 hr). Initial moisture content is determined gravimetrically and expressed on a fresh weight basis after drying for 3 days at 90°C.

Scions are cut into 1 bud (35 mm sections) and allowed to dry on screens in a -5°C cold room or freezer. When sections reach 25% (30% for apple) moisture they are wrapped in plastic and held at -5°C. For cooling, 10 sections are sealed in a polyolefin tube and heat sealed with wooden plugs at each end.

Samples are cooled in a liquid nitrogen-pulsed chamber (1°C/hr) from -5°C to -30°C. After 24-48 hr the tubes are transferred to the vapor-phase LN_2 for long-term storage. Warming occurred by transfer of tubes to a + 4°C cold room where they are held overnight. Sections were removed from the tube and rehydrated in peat moss for 15 d at 2°C. Recovered buds were chip budded to 1 yr old seedling rootstocks.

Dormant Bud Vitrification for Cold-hardy Shoots

Matsumoto, T; Mochida, K; Itamura, H; Sakai Cryopreservation of persimmon (Diospyros kaki Thunb.) by vitrification of dormant shoot tips, Plant Cell Reports, 2001: 20: 398-402.

1. Dormant axillary buds collected in January. 50 cm twigs of 1 year wood with about 20 buds per twig were sealed in a plastic bag and stored at -1 °C.
2. 5 cm sections with 2-3 buds were washed in soapy water, sterilized in 70% alcohol for 2 min and in 2% sodium hypochlorite solution with 0.01% Tween 20 for 20 min.
3. Rinsed 3X in sterile water and the 1mm shoot tips dissected. Shoot tips consisted of about 5 leaf primordia
4. Preculture on MS 0.3 M sucrose medium with ½ x nitrogen and 0.2% gellan gum for 1 day at 25 °C
5. Use loading solution for 20 min at 25 °C (2 M glycerol + 0.4 M sucrose).
6. PVS2 for 20 min at 25 °C
7. LN
8. Rapid warming in 40 °C water for 2 min.
9. Rinse in 1.2 M sucrose MS for 20 min at 25 °C.
10. Use a filter paper between the meristem and the recovery medium and transfer to new medium after one day by moving the filter paper.

APPENDICES:

RECIPE SHEETS FOR CRYOPRESERVATION SOLUTIONS

Liquid medium for use with slow-cooling technique

Murashige and Skoog

Component	Conc./L	Amount Needed
M & S Nitrates	10 ml	
M & S Sulfates	10 ml	
M & S Halides	10 ml	
M & S P, B, Mo	10 ml	
M & S Iron	10 ml	
M & S Vitamins	10 ml	
Sucrose	30 g	

Bring to final volume

pH 5.7

Divide into containers with about 50 – 75 ml per container and autoclave.

DMSO pre-treatment plates for slow-cooling and vitrification techniques

Murashige and Skoog 1962

Component	Conc./L	Amount Needed
M & S Nitrates	10 ml	
M & S Sulfates	10 ml	
M & S Halides	10 ml	
M & S P, B, Mo	10 ml	
M & S Iron	10 ml	
M & S Vitamins	10 ml	
Sucrose	30 g	

Bring to final volume

pH 5.7

Cryopreservation pretreatment Petri dishes

Agar only 8 g

Or you can use a mixture of agar and Gelrite

Agar 3.5 g
and
Gelrite 1.75 g

Autoclave in a flask and while still warm

Add DMSO (5%) 50 ml

Pour plates in the laminar flow hood

Sucrose pre-treatment plates

0.3 M sucrose pretreatment medium for vitrification
0.75M sucrose pretreatment for Dumet E-D
Murashige and Skoog 1962

Component	Conc./L	Amount Needed
M & S Nitrates	10 ml	
M & S Sulfates	10 ml	
M & S Halides	10 ml	
M & S P,B,Mo	10 ml	
M & S Iron	10 ml	
M & S Vitamins	10 ml	
Sucrose MW 342.3		
Sucrose (0.3 M)	102.7 g	
or		
Sucrose (0.75M)	256.73 g	
Bring to final volume		
pH	5.7	

Cryopreservation pretreatment Petri dishes

Agar only	8g

Or you can use a mixture of agar and Gelrite

Agar	3.5 g
and	
Gelrite	1.75 g

High-Sucrose Medium for Vitrification Procedure

If your autoclave has a cycle longer than 40 minutes it may caramelize the sugars so you will need to filter sterilize these solutions.

0.8 M sucrose is for making vitrification solutions

1.2 M sucrose medium is for rinsing meristems after thawing.

Make 1 L of base MS with no vitamins or hormones and add extra sucrose as shown.

Murashige and Skoog 1962

Stocks Component medium	Conc./L	0.8 M Sucrose medium	1.2 M Sucrose
M & S Nitrates	10 ml		
M & S Sulfates	10 ml		
M & S Halides	10 ml		
M & S P,B,Mo	10 ml		
M & S Iron	10 ml		
		Bring to 500 ml volume (2X medium)	
Divide to half volume		250 ml	250 ml
Sucrose MW 342.3			
0.8 M Sucrose	273.84g	136.92	none
1.2 M Sucrose	410.76 g	none	205.38
Bring to final volume		500 ml	500 ml

pH 5.7

Put medium in 125 ml flasks (75 ml each) or screw top bottles.

Cap with foil and autoclave, cool before using
Can be kept in the refrigerator for several months after autoclaving.

LS Loading Solution for Vitrification (Sakai et al.)

2 M glycerol in 0.4 M sucrose MS medium
Glycerol MW 92

To make 100 ml of loading solution:

Take 50 ml of 0.8M sucrose MS medium
add 18.4 grams glycerol
Bring to 100 ml volume with distilled water.

Alginate Bead Medium

For liquid alginate solution for making beads
beads.doc
4-22-99

This medium must not have any calcium, so leave out the halide stocks. Make 250 to 500 ml at one time and store the extra in the refrigerator.

Component	Conc./L	Amount Needed
M & S Nitrates	10 ml	
M & S Sulfates	10 ml	
M & S P, B, Mo	10 ml	
M & S Iron	10 ml	
Sucrose (0.75M)	256.73 g	

Bring to final volume

pH 5.7

Before heating, and with stirring, slowly (very slowly, do not let it make lumps) add
Alginic Acid (2% low viscosity) 30 g/L

After it is all dispersed in the liquid, then heat. If you heat first it will form an insoluble ball and will be useless. It will not melt in the autoclave unless it is well dispersed first.
Autoclave in 125 or 250 ml flasks (half filled) and foil covered

Alginate Bead 0.75 M Sucrose Medium and $CaCl_2$ Medium

0.75 M sucrose medium is for overnight conditioning of the beads or with agar for pretreatment of shoot tips
$CaCl_2$ medium is used for forming the beads

Make 1 L of MS without hormones and divide as follows:

Murashige and Skoog 1962 Stocks		500 ml 0.75 ml Sucrose	500 ml 100 mM CaCl2
Component Needed	Conc./L	Amount Needed	Amount
M & S Nitrates	10 ml		
M & S Sulfates	10 ml		
M & S Halides	10 ml		
M & S P,B,Mo	10 ml		
M & S Iron	10 ml		
M & S Vitamins	10 ml		
Sucrose	30 g		
		Make up to 500 ml (2X medium)	
and divide into two parts		250 ml	250 ml
Sucrose MW 342.3 Extra Sucrose		141.15 g/500ml	none
CaCl2 g/500 ml		none	7.3
Bring to final volume		500 ml	500 ml
pH 5.7			

Put 0.75 M sucrose medium in 125 ml flasks (75 ml each)

Put calcium chloride solution in 250 ml or larger flasks (100 ml/250 ml flask)

Cap with foil and autoclave, cool before using

Standard MS Medium (use as liquid medium for rehydrating alginate beads)

Murashige and Skoog 1962

Component	Conc./L	Amount Needed
M & S Nitrates	10 ml	
M & S Sulfates	10 ml	
M & S Halides	10 ml	
M & S P,B,Mo	10 ml	
M & S Iron	10 ml	
M & S Vitamins	10 ml	
Sucrose	30 g	

Add growth regulators as needed

Bring to final volume

pH 5.7

Agar only 7g

Or you can use a mixture of agar and Gelrite

Agar 3.5 g
and
Gelrite 1.35 g

Revised 6-6-03CP

MURASHIGE AND SKOOG MEDIUM STOCK SOLUTIONS

1962 STOCK SOLUTIONS
FOR 1 L STOCK SOLUTIONS AT 100X

SOLUTION	COMPONENT	FORMULA	AMT/L
1. NITRATES	Ammonium Nitrate	NH_4NO_3	165 g
Do not	Potassium Nitrate	KNO_3	190 g
Refrigerate			
2. SULFATES	Magnesium Sulfate	$MgSO_4*7H_2O$	37 g
Refrigerate	Manganous Sulfate	$MnSO_4*H_2O$	1.69 g
	Zinc Sulfate	$ZnSO_4*7H_2O$	860 mg
	Cupric Sulfate	$CuSO_4*5H_2O$	2.5 mg
3. HALIDES	Calcium Chloride	$CaCl_2*2H_2O$	44 g
Refrigerate	Potassium Iodide	KI	83 mg
	Cobalt Chloride	$CoCl_2*6H_2O$	2.5 mg
4. POTASSIUM	Potassium Phosphate	KH_2PO_4 (monobasic)	17 g
BORON	Boric Acid	H_3BO_3	620 mg
MOLYBDENUM	Sodium Molybdate	$NaMoO_4*2H_2O$	25 mg
Refrigerate			
5. IRON	Ferrous Sulfate	$FeSO_4*7H_2O$	2.79 g
Refrigerate	Sodium EDTA	$Na_2EDTA*2H_2O$	3.73 g
Light Sensitive			
(heat until the solution turns a rich yellow then cool and store in a foil wrapped bottle)			
6. M&S VITAMINS	Thiamine HCL		40 mg
Freeze in 10-15 ml aliquots	Glycine		200 mg
	Myo-inositol		10 g
	Nicotinic acid		50 mg
	Pyridoxine HCL		50 mg
7. THIAMINE STOCK	Thiamine HCL		100 mg
Freeze in 10-15 ml aliquots			

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